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Identification of oxidized low density lipoprotein in human renal biopsies[1]

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Identification of oxidized low density lipoprotein in human renal biopsies.

Background. Intraglomerular lipid deposition is frequently observed in routine renal biopsies, and it has been suggested that lipid peroxidation of low density lipoprotein (LDL) may be implicated in the pathogenesis of progressive glomerulosclerosis. We have examined whether oxidized LDL (Ox-LDL) is present in the glomeruli of patients with renal disease and whether intrinsic human glomerular cells express NADPH-oxidase (a superoxide-generating enzyme found in professional phagocytes).

Methods. Immunocytochemical study was performed on 939 renal biopsy specimens, using monoclonal antibodies (mAbs) OL-10, 48 and 449, and polyclonal antiobody against human apolipoprotein (apo) B. Mouse mAb OL-10 recognizes malondialdehyde (MDA)-modified peptide epitope, and mAbs 48 and 449 react with α and β subunits of cytochrome *b*558, an essential component of NADPH-oxidase.

Results. Sixty-two (6.6%) of the 939 patients with renal disease exhibited a staining for MDA-altered protein or Ox-LDL in the glomeruli, mainly in the sclerotic segments or mesangial areas. Group 1 patients with heavy Ox-LDL deposition mainly in the sclerotic segments showed a higher frequency of renal insufficiency and heavy proteinuria and a greater degree of glomerulosclerosis, compared to those in group 2 with mesangial Ox-LDL staining. The distribution of MDA protein epitopes, in general, paralleled the deposition of apo B epitopes. Immunoelectron microscopy of ultrathin frozen sections showed the presence of immunogold particles for mAbs 48 and 449 in the cytoplasm of resident glomerular cells of both normal and diseased kidneys. When immunoblotted with mAb OL-10, one band from the IgA nephropathy and focal segmental glomerulosclerosis groups at ~260 kD was labeled, whereas immunostaining of normal control samples revealed no staining.

Conclusions. These results indicate that Ox-LDL is present mainly in the lesions of glomerulosclerosis and mesangial areas in human renal biopsies. They also suggest that patients with heavy Ox-LDL accumulation in the sclerotic segments of glomeruli have more advanced renal disease than those with mesangial Ox-LDL and that resident glomerular cells generate cytochrome *b*558, the potential of which may not suffice to induce peroxidation of LDL in the diseased glomeruli.

Superoxide (O_2^-) or other reactive oxygen species (ROS) are known to be involved in the mediation of renal injury [1, 2]. ROS cause lipid peroxidation of low density lipoprotein (LDL), which results in derivatization of lysine residues of apolipoprotein (apo) B by lipid peroxide breakdown products, such as malondialdehyde (MDA) and 4-hydroxynonenal [3]. Studies have suggested that oxidized LDL (Ox-LDL) may be implicated in the pathogenesis of progressive glomerulosclerosis [4–8]. In support of this view, the presence of Ox-LDL has been demonstrated in the glomeruli of rats with focal segmental glomerulosclerosis (FSGS) [9, 10]. Furthermore, dietary antioxidant reduced not only the staining intensity of Ox-LDL but also the severity of renal injury in this animal model [10].

In a previous study, we reported that apo B-containing lipoproteins were frequently accumulated in the glomeruli of patients with various glomerular diseases [11]. It is conceivable that locally accumulated apo B-containing lipoproteins in the glomeruli provide polyunsaturated fatty acids as substrates for lipid peroxidation. To date, however, Ox-LDL has not been immunohistochemically demonstrated in the glomeruli of renal patients [12]. Only weakly immunoreactive hypochlorite (HOCl)-modified proteins have been demonstrated in the glomeruli of patients with nephrosclerosis, but not in those with minimal lesion or FSGS [13].

The source of ROS in the glomeruli of renal patients is unclear. NADPH-oxidase complex is the most familiar source of superoxide generated for bacterial killing in neutrophils [14, 15]. The same oxidase is believed to be present in all the professional phagocytes, including monocytes/macrophages [16]. This enzyme complex catalyzes the transfer of electrons from NADPH via cytochrome *b*558 to oxygen [14–16]. Radeke et al first demonstrated that cultured human mesangial cells, nonphagocytes, express the NADPH-oxidase complex [17]. In addition, glomerular

[1] **See *Editorial* by Gröne, p. 995.**

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epithelial cells and mesangial cells of rats with passive Heymann nephritis (PHN) were found to express cytochrome *b*558 [18]. No study, however, has identified the NADPH-oxidase system in human glomeruli *in situ*.

The present study was designed to determine whether Ox-LDL is present in human renal biopsies and whether intrinsic glomerular cells express the ROS-generating system. We demonstrated that MDA-altered protein, which co-localized with apo B-containing lipoprotein, occurred mainly in the FSGS lesions and mesangial areas of patients with renal disease and that resident glomerular cells generated cytochrome *b*558.

METHODS

Antibodies

Mouse monoclonal antibody, mAb OL-10, was raised against human Cu^{++}-catalyzed Ox-LDL and was specific for the MDA-modified peptide epitope, as we have previously described [7]. Mouse mAb 449, which reacted with the low molecular weight α-subunit of 22 to 23 kD, and mAb 48, which reacted with the high molecular weight β-subunit of 75 to 90 kD of purified neutrophil cytochrome *b*558 [19], were kindly donated by Dr. Dirk Roos, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service. Goat anti-apo B and rhodamine-conjugated rabbit anti-goat IgG were supplied by Chemicon (Temecula, CA, USA); mouse anti-human CD68, FITC-labeled rabbit anti-mouse immunoglobulin, and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin were purchased from Dakopatts (Glostrup, Denmark), and FITC-labeled rabbit anti-goat IgG from Kallestad (Austin, TX, USA). Affinity-purified goat anti-mouse IgG-10 nm-gold was obtained from Amersham (Arlington Heights, IL, USA).

Pathological study

A total of 939 renal biopsies having more than ten glomeruli were subjected to light, electron, and immunofluorescence microscopy using standard methodologies. By light microscopy, segmental sclerosis was defined as segmental collapse and solidification of glomeruli associated with excess mesangial matrix accumulation. Global sclerosis was defined as total collapse with solidification involving the whole glomerulus. The term "glomerulosclerosis" was used to describe both segmental sclerosis and global sclerosis, as described [20].

Normal control specimens were obtained from completely normal regions of four nephrectomy specimens that elsewhere contained renal cell carcinoma, and from four renal biopsy specimens at the time of donation for kidney transplantation.

Immunofluorescence microscopy

Biopsy specimens were embedded in Tissue-Tek O.C.T. compound, immersed in cooled isopentane, and snap frozen in liquid nitrogen. Cryostat sections were fixed for 10 minutes in freshly prepared 4% paraformaldehyde, 20 μM butylated hydroxytoluene (BHT), 2 mM EDTA, and 5% sucrose. These were rinsed three times for five minutes in 0.1 M phosphate buffered solution (PBS), pH 7.4, containing 20 μM BHT, 2 mM EDTA, and 5% sucrose. To detect MDA-altered protein, human macrophage CD68, or cytochrome *b*558, sections were incubated with either mAb OL-10 (2 μg/ml), anti-CD68 (1:50), mAb 449 (2.5 μg/ml) or mAb 48 (2.5 μg/ml) for one hour at 25°C, followed by FITC-labeled rabbit anti-mouse immunoglobulin (1:50). The staining method used to detect apo B epitopes has been previously described [11]. Positive tissue control for MDA-altered protein or Ox-LDL was obtained from aorta sections containing fibro-fatty atherosclerotic lesions at the time of autopsy. Negative control studies were performed by omitting the primary antibodies or replacing them with corresponding non-immune serum. Sections were mounted in 1% *p*-phenylene-diamine-glycerol, pH 8.0, and examined with a Zeiss fluorescence microscope.

For double immunostaining, two primary antibodies, mAb OL-10 and goat anti-apo B (1:220), were used. After reaction with primary antibodies, the sections were exposed to two secondary antisera, FITC-labeled rabbit anti-mouse immunoglobulin and rhodamine-conjugated rabbit anti-goat IgG (1:200).

Specificity for the staining of MDA-modified proteins was further confirmed by absorption test and summarized below: mAb OL-10 with a staining titer of 1:2 was diluted twofold in buffer and mixed with an equal volume of 500 μg/ml Cu^{++}-catalyzed-Ox-LDL or MDA-altered LDL. Cu^{++}-catalyzed Ox-LDL and MDA-altered LDL were prepared, as we have previously described [7]. After incubation at 37°C for one hour, the mixture was centrifuged at 4°C, at 9,000 rpm for 30 minutes, and the supernatant was tested for the ability to stain MDA-altered proteins.

Immunogold electron microscopy

Small (1 mm^3) pieces of the renal biopsy were immersed in 2% paraformaldehyde and 0.2% glutaraldehyde for two hours at 4°C, washed twice for five minutes at 4°C with 0.1 M PBS, infiltrated with 2.3 M sucrose for more than one hour and frozen in liquid nitrogen. Ultrathin frozen sections were prepared using a Reichert FCS low-temperature sectioning system, and these were mounted on Formvar-coated 100-mesh nickel grids. These were incubated overnight with either mAb 449 (0.6 μg/ml) or mAb 48 (0.6 μg/ml). Excess unbound primary antibody was washed off the grids using PBS containing 1% bovine serum albumin and 0.05% Tween-20, as we have previously described [21]. The grids were then exposed to 10 nm gold-labeled goat

anti-mouse IgG (1:30) for two hours at room temperature, and washed first with PBS and then with distilled water. Afterwards, the sections were stained for 10 minutes with uranyl acetate and for five minutes with lead citrate, and observed with a Hitachi 7100 electron microscope. For negative controls, the primary antibody was omitted or replaced by corresponding non-immune serum.

Protein electrophoresis and Western blots

We chose the groups of IgA nephropathy and FSGS with sufficient amounts of frozen tissue for Western blot analysis of MDA-altered protein. Frozen renal biopsy specimens from two disease groups were pooled in buffer A containing 0.15 M NaCl and 0.24 mM EDTA, pH 7.4, sonicated and dissolved in 0.2 N NaOH. Samples and Cu^{++}-catalyzed Ox-LDL were electrophoretically resolved in 5% polyacrylamide gel in sodium dodecyl sulfate (SDS) buffer under reducing conditions and transferred onto nitrocellulose membrane. The blots were incubated in blocking solution for one hour and incubated with mAb OL-10 at room temperature for one hour. For competition experiments, mAb OL-10 preabsorbed with excess Cu^{++}-catalyzed Ox-LDL was used. Bound murine antibody was visualized by the subsequent incubation with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin and enhanced chemiluminescence detection kit (Amersham).

Statistics

The significance of differences between groups was obtained using a two-tailed Student's *t*-test and the Chi-square test. All differences were considered significant for $P < 0.05$. Results were expressed as means ± SD.

RESULTS

Characteristics of patients

Of the 939 biopsies, 62 (6.6%) showed positive staining for Ox-LDL in the glomeruli. Patients were grouped according to pathologic diagnosis in Table 1, in which the frequency of Ox-LDL deposition is shown. The diagnoses included idiopathic FSGS (14%), post-transplantation FSGS (43%), IgA nephropathy (5%), membranoproliferative glomerulonephritis (10%), crescentic glomerulonephritis (29%), diabetes mellitus (13%), hypertensive nephropathy (27%), and end-stage renal disease (33%).

The 62 patients with positive staining for Ox-LDL were also grouped according to its intraglomerular localization: group I had Ox-LDL deposition in the sclerotic segments, crescents or subendothelial areas, and totaled 27 patients (44%); group 2's staining was mainly in the mesangial areas, and totaled 35 patients (56%). Clinical data of group 1 at the time of biopsy are summarized in Table 2. Hypercholesterolemia, defined as a cholesterol level greater than 240 mg/dl, was observed in 10 patients (37%). Renal insufficiency, diagnosed as serum creatinine greater than 1.5 mg/dl or as creatinine clearance lower than 80 ml/min/1.73 m^2, was noted in 20 patients (74%). Proteinuria was present in all with the amounts excreted ranging from 0.4 to 8 g per day; in 16 patients (59%), the amount was ≥3.5 g per day. All patients in group 1 showed glomerulosclerosis affecting 12% to 92% (mean 58% ± 31%) of the glomeruli (Table 2).

Table 1. Frequency of intraglomerular oxidized low density lipoprotein deposition in renal biopsies

Diagnosis	No. examined	No. of positive cases (%)
Minimal lesion	131	3 (2.3)
FSGS	85	12 (14.1)
FSGS, recurrent	7	3 (42.9)
IgA nephropathy	272	14 (5.1)
Acute postinfectious GN	39	1 (2.6)
Membranous nephropathy	77	1 (1.3)
Membranoproliferative GN	40	4 (10.0)
Mesangial proliferative GN, nonspecific	55	2 (3.6)
Diffuse crescentic GN	7	2 (28.6)
Systemic lupus erythematosus	75	7 (9.3)
Diabetes mellitus	16	2 (12.5)
Henoch-Schönlein nephritis	25	2 (8.0)
Tubulointerstitial disease	23	1 (4.3)
Hypertensive nephropathy	15	4 (26.7)
Transplant nephropathy	42	2 (4.8)
End-stage renal disease	6	2 (33.3)
Miscellaneous	24	0
Total	939	62 (6.6)

Abbreviations are: FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; No., number.

Clinical data of group 2 are summarized in Table 3. Hypercholesterolemia was present in 11 patients (31%), and renal insufficiency in 17 (49%). Proteinuria was observed in 33 patients with the amounts excreted ranging from 0.3 to 14 g per day; in 10 (29%), the amount was ≥3.5 g per day. Twenty-two cases exhibited glomerulosclerosis affecting 1% to 86% (mean 18% ± 18%) of the glomeruli (Table 3).

In group 1, the frequency of renal insufficiency and heavy proteinuria, as well as the percentage of glomeruli with glomerulosclerosis were significantly higher as compared with those in group 2 ($P < 0.05$ or $P < 0.01$).

Localization of MDA-altered proteins and apo B in human glomeruli

Sixty-two (6.6%) of the 939 renal biopsies exhibited staining for MDA-altered protein or Ox-LDL in the glomeruli by indirect immunofluorescence. Twenty-two of them showed localized but heavy accumulation of Ox-LDL in the sclerotic segments of glomeruli (Fig. 1 A, B), where staining for apo B epitopes was also present. Four patients diagnosed with either crescentic glomerulonephritis, systemic lupus erythematosus, or Henoch-Schönlein nephritis exhibited localization of Ox-LDL in crescents. In a case with membranoproliferative glomerulonephrtis, clumpy

Table 2. Characteristics of patients with oxidized low density lipoprotein in sclerotic lesions or crescents of glomeruli

Diagnosis	*N*	Sex (M:F)	Age *years*	Cholesterol *mg/dl*	C_{Cr} *ml/min/1.73m²*	Proteinuria *g/day*	Glomerulosclerosis %
FSGS	6	2:4	32 ± 13	251 ± 86	57 ± 28	4.9 ± 2.0	61 ± 35
FSGS, recurrent	2	1:1	13, 18	432, 339	31, 38	4.0, 3.9	75, 55
IgA nephropathy	7	4:3	34 ± 22	229 ± 84	62 ± 57	2.5 ± 1.7	47 ± 35
Membranoproliferative GN	2	1:1	17, 49	120, 317	37, 120	1.1, 3.6	12, 50
Crescentic GN	2	2:0	74, 53	183, 177	18, 31	1.6, 2.3	68[a], 82[a]
HSN	1	0:1	8	249	80	2	22
SLE	3	0:3	26 ± 4	354 ± 148	26 ± 20	6.4 ± 0.8	86 ± 5
Diabetes mellitus	2	2:0	52, 67	206, 167	54, 52	5.8, 0.8	58, 20
End-stage renal disease	2	2:0	24, 28	198, 153	9, 29	7.2, 1.3	91, 79
Total	27	14:13	34 ± 19	251 ± 103	49 ± 37	3.7 ± 2.2	58 ± 31

Abbreviations are: C_{Cr}, creatinine clearance; FSGS, focal segmental glomerulosclerosis; GN, glomerulonephritis; HSN, Henoch-Schönlein nephritis; SLE, systemic lupus erythematosus. Values are mean ± SD.

[a] Including % of glomeruli with crescents

Table 3. Characteristics of patients with oxidized low density lipoprotein in mesangial areas of glomeruli

Diagnosis	*N*	Sex (M:F)	Age *years*	Cholesterol *mg/dl*	C_{Cr} *ml/min/1.73/m²*	Proteinuria *g/day*	Glomerulosclerosis %
Minimal lesion	3	3:0	23 ± 9	475 ± 43	83 ± 27	7.8 ± 4.8	1 ± 2
FSGS	6	5:1	31 ± 24	195 ± 43	82 ± 49	2.1 ± 1.3	25 ± 17
FSGS, recurrent	1	0:1	58	478	43	14.0	86
IgA nephropathy	7	5:2	27 ± 12	181 ± 56	86 ± 30	1.8 ± 1.1	27 ± 11
Acute postinfectious GN	1	0:1	22	248	30	6.9	20
Membranous nephropathy	1	1:0	42	246	82	3.8	7
Membranoproliferative GN	2	2:0	17, 48	271, 319	29, 77	1.2, 8.5	5, 4
Mesangial proliferative GN, nonspecific	2	1:1	16, 58	139, 163	141, 67	1.2, 0.5	0, 2
Systemic lupus erythematosus	4	0:4	35 ± 3	206 ± 52	82 ± 20	3.0 ± 1.8	15 ± 12
Henoch-Schönlein nephritis	1	1:0	9	186	101	0.7	0
Tubulointerstitial disease	1	0:1	43	176	76	0	0
Hypertensive nephropathy	4	3:1	48 ± 13	200 ± 33	72 ± 30	1.3 ± 1.1	18 ± 10
Transplant nephropathy	2	2:0	39, 30	233, 215	45, 56	0, 0.9	0.2, 0
Total	35	23:12	32 ± 16	235 ± 110	78 ± 31	3.2 ± 3.4	18 ± 18

Values are mean ± SD. Abbreviations are in Table 2.

deposition of Ox-LDL occurred in subendothelial areas (Fig. 1C). In the remaining 35 patients, intracellular and/or extracellular deposition of Ox-LDL was present mainly in mesangial areas (Fig. 1D). Surprisingly, six (75%) of the eight normal controls and many biopsy samples showed weak staining for the MDA-specific epitope in the periphery of the glomerular capillary loops (Fig. 1E). In this area, however, staining for apo B epitopes was negative. The specificity of staining for MDA-altered protein was confirmed by an absorption test (Fig. 1F).

Dual immunofluorescence of the same section of kidney, using mAb OL-10 and anti-apo B, demonstrated co-localization of reactive epitopes in the mesangial areas or sclerotic segments of glomeruli (Fig. 2 A–C).

Also, 85 (9.1%) of the 939 renal biopsies showed localization of MDA-altered protein in intimal or subendothelial layer and media of interlobular arteries (Fig. 3).

All three patients with post-transplantation recurrent FSGS and intraglomerular Ox-LDL deposition had more than one post-transplantation renal biopsy. In first or previous biopsy samples, showing a lower percentage of the glomeruli with sclerosis compared to the current ones, no staining for Ox-LDL was demonstrable.

Localization of cytochrome *b*558 in human glomeruli

Indirect immunofluorescence study was performed using mAbs 48 and 449 on 174 biopsies. Forty-five cases (25.9%) showed positive staining mainly in cells in the mesangium. In the glomeruli of patients with IgA nephropathy or postinfectious glomerulonephritis, they showed a segmentally distributed, punctate, cytoplasmic pattern (Fig. 4). These cells frequently matched for CD68-positive cells. In some cases with membranous nephropathy and minimal lesion, which showed no infiltration of neutrophils or monocytes, patchy cytoplasmic staining for mAbs 48 and 449 was rarely seen in glomerular epithelial cells and in mesangial cells.

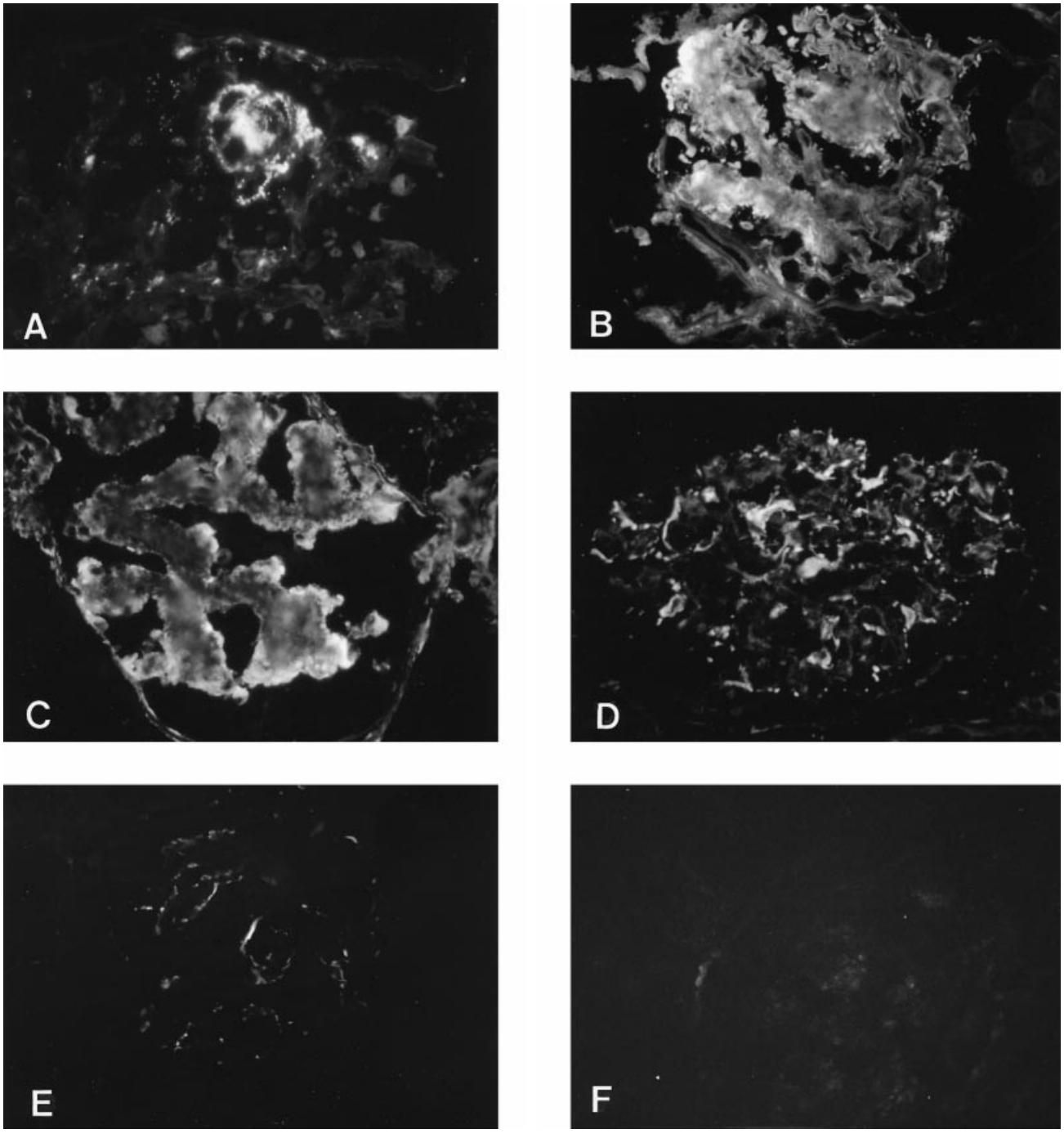

Fig. 1. Localization of malondialdehyde (MDA) protein epitopes by immunofluorescence in glomeruli of patients with focal segmental glomerulosclerosis (FSGS) (*A, D*), diabetes mellitus (*B*), and membranoproliferative glomerulonephritis (*C*), and of a normal control (*E*). Localized heavy staining for MDA-altered protein is shown in the sclerotic segments (A, B), subendothelial areas (C), or mesangial areas (D) of glomeruli. In contrast, only specks for MDA protein epitopes are noted in the periphery of glomerular capillary loops of a normal control (E). A parallel section to that shown in (A), showing no staining for MDA epitopes when mAb OL-10 was preabsorbed with excess MDA-altered LDL (F). Original magnification, ×400.

By immunoelectron microscopy of ultrathin frozen sections, binding of mAbs 48 and 449 was noted in the cytoplasm of intrinsic glomerular cells of both normal controls and patients with renal disease (Fig. 5). Particle labeling against cytochrome *b*558 was scattered in the cytoplasm; it was not specifically localized in intracellular vesicles. There was no labeling for cytochrome *b*558 within the immune deposits. As expected, more gold particle

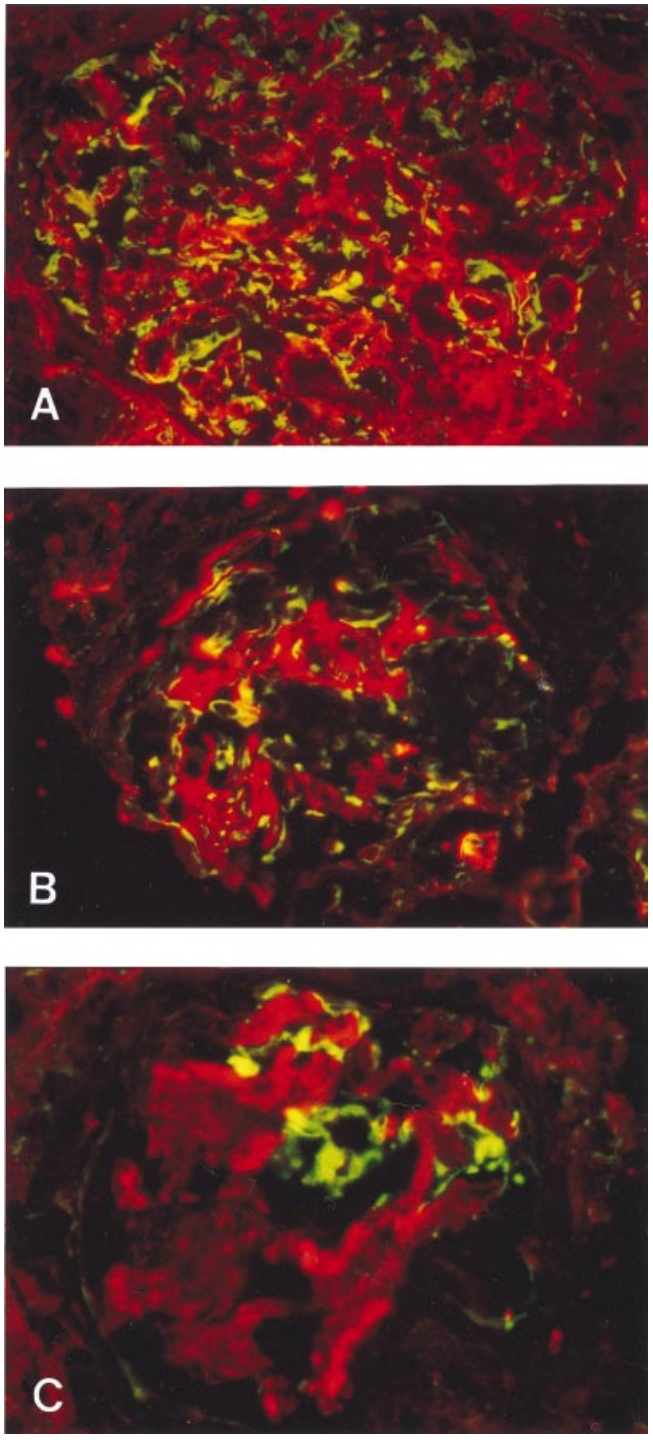

Fig. 2. Dual immunofluorescence of glomeruli from patients with focal segmental glomerulosclerosis (FSGS) (*A, C*), and IgA nephropathy (*B*). The same glomerular sections were stained with mAb OL-10 (FITC) and anti-apolipoprotein (apo) B (rhodamine). When both fluorochromes are viewed simultaneously, co-localization of MDA-altered protein and apo B-containing lipoprotein can be appreciated in mesangial areas (A), and in the lesions of glomerulosclerosis (B,C). Original magnification, ×400. Reproduction of this figure in color was made possible by a grant from Cheil Jedang Corporation, Seoul, Korea.

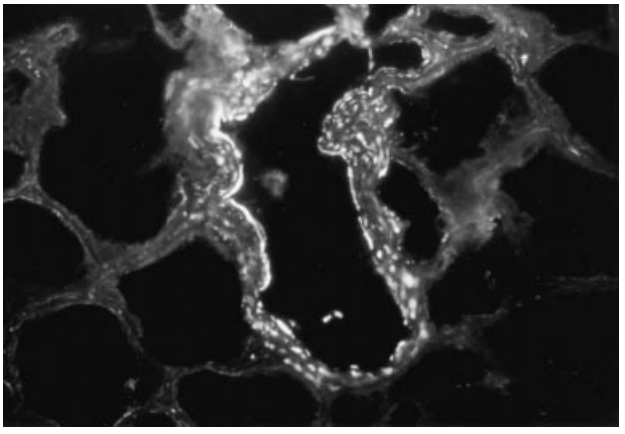

Fig. 3. Localization of malondialdehyde (MDA)-altered protein by immunofluorescence in intimal or subendothelial layer and media of a preglomerular artery. Original magnification, ×400.

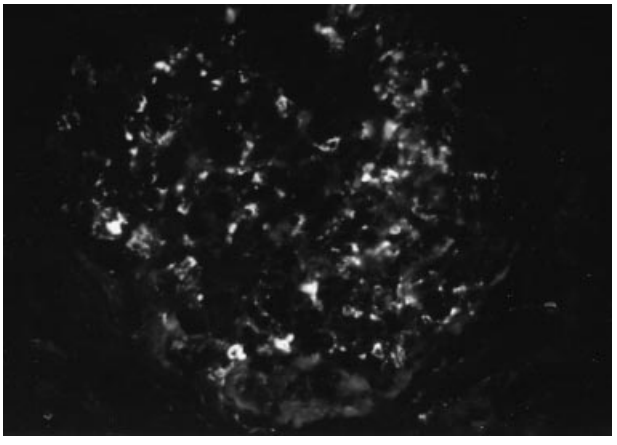

Fig. 4. Localization of cytochrome *b*558 by immunofluorescence in a glomerulus of a patient with IgA nephropathy. Segmental cytoplasmic staining for cytochrome *b*558 is seen in mesangial area. Original magnification, ×400.

density for cytochrome *b*558 was found within polymorphonuclear leukocytes present in capillary lumen than within glomerular cells.

Western blot analysis of MDA-altered protein in renal biopsies

Pooled renal biopsy samples from IgA nephropathy and FSGS groups, normal control samples and Cu^{++}-catalyzed Ox-LDL were dissolved in SDS-sample buffer, and 22 μg or 38 μg of protein from each preparation was subjected to SDS-gel electrophoresis and immunoblotting. When immunoblotted with mAb OL-10, one band from the two disease groups at ~260 kD was labeled, whereas immunostaining of normal control samples revealed no staining. Immunoblotting of Ox-LDL with mAb OL-10 showed that two proteins of approximate molecular weight ~240 kD and ~260 kD were immunoreactive (Fig. 6). Competition experiments with mAb OL-10 preabsorbed with Ox-LDL

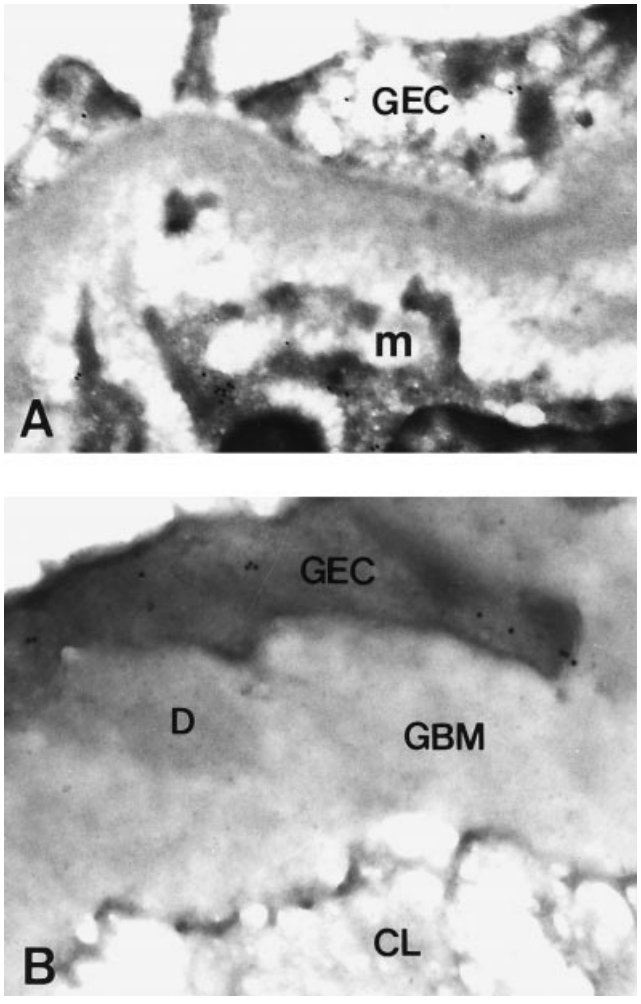

Fig. 5. Electron micrograph of gold-labeled antibody to cytochrome *b*558, mAb 449, in a glomerulus of a normal control (*A*) and a patient with membranous nephropathy (*B*) showing the distribution of gold particles in the cytoplasm of glomerular epithelial cells (GEC) and mesangial cells (m). Abbreviations are: GBM, glomerular basement membrane; D, subepithelial electron-dense deposits; CL, capillary lumen. (A) ×33,000; (B) ×46,000.

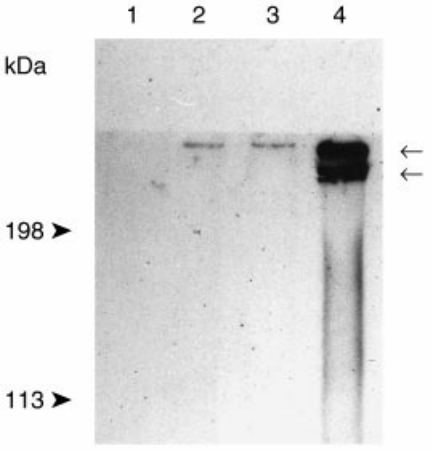

Fig. 6. Identification of malondialdehyde (MDA)-modified protein in lysates of renal biopsy samples by immunoblotting. Samples (38 μg protein/lane, lanes 1 to 3) and Cu^{++}-catalyzed Ox-LDL (22 μg protein/lane, lane 4) were separated on 5% polyacrylamide gel in SDS buffer under reducing conditions and transferred onto nitrocellulose. Mouse mAb OL-10 bound to a protein of approximate molecular weight ~260 kD in samples of IgA nephropathy (lane 2) and FSGS (lane 3), whereas no staining was seen in the normal control samples (lane 1). In Ox-LDL (lane 4), two proteins of approximate molecular weight ~240 kD and ~260 kD were immunoreactive with mAb OL-10.

prevented antibody binding, indicating that staining was specific for MDA-modified protein.

DISCUSSION

The main finding of this study is that Ox-LDL is present in human renal biopsies. We demonstrated by immunofluorescence the presence of MDA-altered protein, which co-localized with apo B-containing lipoprotein, mainly in the lesions of FSGS and mesangial areas.

The occurrence of MDA-modified protein within the lesions of FSGS is similar to that described within atheroma of Watanabe heritable hyperlipidemic rabbits [22–24] and supports the hypothesis that glomerulosclerosis is analogous to atherosclerosis [4, 25, 26]. Co-localization of MDA-altered protein and apo B-containing lipoprotein, in particular, suggests that LDL is main substrates for lipid peroxidation and that Ox-LDL is formed in the diseased glomeruli.

The frequency of intraglomerular Ox-LDL deposition in this study is 6.6%, which is approximately one-fourth of that of intraglomerular apo B deposition [11]. This suggests that LDL trapped in the glomeruli of renal patients, in general, might be refractory to oxidative modification. Or Ox-LDL synthesized *in vivo* might be rapidly degraded or removed [27]. The degree of oxidation determines the degree of recognition of Ox-LDL by scavenger receptors [28]. If less severely oxidized LDL is formed in the glomeruli, it might be taken up by monocytes/macrophages, leading to formation of foam cells. Even mesangial cells expressing scavenger receptors [29] could join this process. The low frequency of intracellular Ox-LDL staining in the mesangium suggests that MDA adducts are rapidly degraded within macrophages or mesangial cells, with decreased antibody reactivity. Or some Ox-LDL products, which may not have common model epitope such as MDA-modified peptide epitope, may not be recognized by mAb OL-10, the only monoclonal antibody used in this study. It is also possible that Ox-LDL formed in the glomeruli might circulate in plasma unless the oxidation is severe [27]. Thus, heavy accumulation of Ox-LDL in the lesions of FSGS or subendothelial areas in this study might reflect that LDL is so severely oxidized that no uptake occurs by either the LDL or scavenger receptors.

Oxidative stress seems to be increased in uremic patients. Plasma from uremic patients contains considerable levels of lipid peroxidation products [30] and autoantibodies against Ox-LDL [31]. Major modifications could occur in

the chemical properties of LDL from patients with uremia and renal transplants, and these modifications would increase susceptibility of LDL to oxidation [32, 33]. In this study, most group 1 patients with heavy deposition of Ox-LDL in the sclerotic segments had advanced glomerulosclerosis accompanied by renal dysfunction, partly supporting this notion.

In PHN, a rat model of human membranous nephropathy, ROS were produced by glomerular epithelial cells [18]. MDA adducts were localized to glomerular epithelial cells, to the glomerular basement membrane and to immune deposits [34]. Furthermore, apo B- and apo E-containing lipoproteins accumulated within immune deposits of PHN rats and were modified by lipid peroxidation [35]. We also demonstrated immunogold particles for cytochrome *b*558 in glomerular epithelial cells of patients with membranous nephropathy and of normal controls. However, the distribution of MDA-protein epitopes in the periphery of glomerular capillary loops did not parallel the deposition of apo B epitopes. This suggests that lipid peroxidation decomposition products in the peripheral loops of human glomeruli are not derived from LDL. The reason why a positivity for MDA-adducts occurred in peripheral rims of normal glomeruli is not clear. It is tempting to speculate that in glomerular epithelial cells, which are not in direct contact with circulation, antioxidant levels may be overwhelmed by the generation of oxidants. Or lipid peroxidation products, which could be present in urinary space, may diffuse into glomerular epithelial cells to be sources of MDA-adducts.

NADPH-oxidase complex is expressed not only in phagocytes but also in nonphagocytes, such as human mesangial cells [17], human fibroblasts [36], murine osteoclasts [37], and bovine vascular smooth muscle cells [38]. Unlike phagocytes, the rate of superoxide production by cultured human mesangial cells, endothelial cells and fibroblasts is low and is not suicidal [17, 36]. Even in nonpurified blood phagocytes, cytochrome *b*558 components are concentrated in secretory vesicles for the formation of a rapidly mobilizable pool [39]. In the present study, intracytoplasmic labeling for these components was rather randomly scattered in the glomerular cells, and this supports the notion that glomerular cells are much less likely than phagocytes to generate ROS.

In view of the relatively limited superoxide-generating potential of intrinsic glomerular cells, monocytes/macrophages might participate in lipid peroxidation of LDL in this study. Whether Ox-LDL in the lesions of FSGS is the cause or effect of glomerulosclerosis is unclear. We only assume that intraglomerular foam cells having internalized Ox-LDL could contribute to the eventual glomerulosclerosis, as originally proposed in atherogenesis [40]. In addition, peroxidative products of LDL stimulated collagen gene expression in cultured human mesangial cells, suggesting that Ox-LDL may be implicated in the development of glomerulosclerosis [7].

In summary, the present study demonstrates that Ox-LDL is present mainly in the lesions of glomerulosclerosis and mesangial areas of patients with renal disease and that intrinsic glomerular cells express cytochrome *b*558, the potential of which may not suffice to be involved in intraglomerular peroxidation of LDL.

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APPENDIX

Abbreviations used in this article are: apo B, apolipoprotein B; FSGS, focal segmental glomerulosclerosis; HOCL, hypochlorite; LDL, low density lipoprotein; mAb, monoclonal antibody; MDA, malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; O_2^-, superoxide; Ox-LDL, oxidized low density lipoprotein; PBS, phosphate buffered saline; PHN, passive Heymann nephritis; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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